International Journal of Biotechnology Research Vol. 5(1), pp. 001-010, August 2018
Available online at http://academeresearchjournals.org/journal/ijbr
ISSN 2328-3505

Full Length Research Paper

Isolation and Screening of *P* -Solubilising Endophytic Diazotropic Bacteria from Ethno-Medicinal Indigenous Rice of Jharkhand

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Accepted 10 August, 2018

Phosphorus is the second important key element after nitrogen as a mineral nutrient in terms of quantitative requirement for rice cultivation. Although abundant in soils, in both organic and inorganic forms, its availability is restricted as it occurs mostly in insoluble forms. Endophytic bacteria are those bacteria that reside inside the plant tissue and directly affect the growth and resistance of the associated plant by the plant growth promoting activity. These bacteria in addition to nitrogen fixation also produce a wide range of growth promoting substances and have the ability to solubilize insoluble form or rock phosphate into soluble form which are easily assessable to the plant growth and yield indigenous varieties which form the basis of pure line for genetic plant breeding at the verge of extinction due to the intervention of hybrid and high yielding variety for increasing productivity. Jharkhand has been the centre of origin of indigenous rice and about 900 varieties are reported to be indigenous to this region. The indigenous and traditional varieties are unique in genotype and harbours within it lots of beneficial bacteria and have many ethnomedicinal properties. But the productivity and the cultivation pattern of these varieties are becoming marginal which is causing huge loss to the rich rice gene pool of the region. In this study four different indigenous rice i.e Sada Gora (SG) ,Bala Gora (BG) ,Kala Gora (KG) and Kharani (KH) with lots of ethnomedicinal properties were taken for the study. Isolation, characterisation of diazotropic endophytic bacteria from these varieties was done. The bacterial isolates were tested for their ability to solubilize phosphate. Out of the 15 diazotropic endophytic isolates 11 isolates were shown to solubilize phosphate.

Keywords: Diazotropic bacterial endophytes, plant growth promoting activity, *p*-solubilization, indigenous rice varieties, gene pool, ethno medicinal value, phytohormones

INTRODUCTION

Jharkhand, land of indigenous tribal people since time immemorial are involved in the cultivating of local and indigenous rice varieties. (Goldstein et al., 1997). Indigenous rice are not only source of dietary requirement and nutritional supplement, rather hold very scared position in their cultural life (Das and Oudhia 2000).

In due course of development, cultivation of indigenous variety has become marginal because of intervention of hybrid and high yielding variety. In the past 15 years there have been major shift in the cultivation pattern of rice from indigenous edaphic climatic adapted variety to High Yielding variety (HYV) and Hybrid Variety (Anonymous 2000) cultivation. Change in cultivation pattern is mainly due to mining and application of heavy fertilizers in hybrid and HYV (Jacqueline and Hart 2001) in rice production which has resulted in huge genetic

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erosion of indigenous rice gene pool and soil nutrient disturbance in the states. In order to make indigenous rice cultivation in Jharkhand sustainable, it is extremely important to combine new microbial technology with traditional knowledge in the area. Bacterial endophytes can offer several benefits to the host plant, particularly growth promotion, protection from pathogens and under diverse environmental conditions.

Bacterial endophytes are able to communicate and interact with the plant more efficiently than rhizospheric bacteria. Identification and screening of growth promoting activities of endophytes with nitrogen fixing ability and show other growth promoting activities (IAA production, siderophore production, phosphate solubilization) can contribute to indigenous rice cultivation and yield improvement (*Krishnan et el., 2014*) .

Jharkhand has about 66% of soils that are low in available phosphorous (Beever and Burns 2000). To satisfy crop nutritional requirements, *P* is usually added to soil as chemical *P* fertilizer; however synthesis of chemical *P* fertilizer is highly energy intensive processes and has long term impacts on the environment in terms of eutrophication, soil fertility depletion, carbon footprint (Shahab and Ahmed 2008). Moreover, plants can use only a small amount of this *P* since 75–90% of added *P* is precipitated by metal–cation complexes, and rapidly becomes fixed in soils (Kim et al., 1997). Such environmental concerns have led to the search for sustainable way of P nutrition of crops. In this regards phosphate-solubilizing bacteria (PSB) have been seen as best eco-friendly means for *P* nutrition of crop (Mohammadi 2012).The use of plant growth promoting bacterial inoculants as live microbial biofertilizers provides a promising alternative to chemical fertilizers and pesticides.

Phosphate solubilizing bacteria (PSB) play very important role in phosphorus nutrition by exchanging its availability to plants through release from inorganic and organic soil phosphorus pools by solubilization and mineralization (Souza et al., 2013). The main mechanism in the soil for mineral phosphate solubilization is by lowering the soil pH by the microbial production of organic acids and mineralization of organic phosphorus by acid phosphates (Panhwar et al., 2012). One of the most common forms of phosphate is fertilizers in the form of rock phosphate or superphosphate (Benson 1998). Hence, bio fertilizers or microbial inoculants are used as an alternate source, which are both economic as well as eco-friendly. Use of phosphate-solubilizing bacteria (PSB) as inoculants has concurrently increased phosphorous uptake in plants and improved yields in several crop species (Barraquio et al., 1997). Inorganic phosphate solubilization is one of the major mechanisms of plant growth promotion by plant associated bacteria (Barraquio et al., 2000).

Jharkhand is the land of indigenous people. Screening of *P* solubilizing endophytic diazotropic bacteria associated with the indigenous rice of Jharkhand will help in increasing the yield potential of indigenous rice grown by poor tribal farmers and will definitely help in improving the cultivation status of the selected rice variety and socio economic condition of the tribal rice cultivating farmers of the state. International Rice Research Institute, Makati city (IRRI) (1981).

MATERIALS AND METHODS

Sources of Endophytic Diazotropic Bacteria For Phosphate solubilization

Indigenous rice varieties Sada Gora, Bala Ggora, Kala Gora and Kharani were chosen for isolation of endophytic diazotrophic bacteria. The selection of these rice varieties for isolation of endophytic bacteria and their important features of the selected varieties and their cultivation pattern are given in Table 1.

Isolation of putative endophytic diazotropic bacteria for *P* solubilisation

Endophytic bacteria were isolated from different parts of selected rice plant such as root and employing standard microbiological methods. Method of Barraquio et al., (1997) was used for isolation (Yadav and Dadarwal 1997). The roots/stems of selected plants were thoroughly washed with tap water to remove all the soil particles before subjecting them to surface sterilization. One gram of roots/stems was taken and washed in triple distilled water (TDW) with sterilized glass beads by vigorous shaking for 3 hr. The plant materials were then dipped in 70% (v/v) alcohol for 3 min followed by three time washings with TDW to remove alcohol. Then they were treated with chloramine-T 1% (w/v) for 15 min and washed thrice with TDW. The last wash was stored and 100 µl of it was plated on NA (nutrient agar) to ensure complete surface sterilization The surface sterilized roots and culms were crushed separately in mortar with pestle and suspended in 1 ml PBS (phosphate buffer saline). The suspension was diluted to 10^{-10} and 100 µl from each dilution was spread on nitrogen-free JNFb$^-$ solid agar-agar medium to enrich N_2-fixing population only. Colonies showing distinct morphological characters were selected and re-cultured on JNFb$^-$ agar medium. All the isolates showing growth on JNFb$^-$ solid medium were also grown in semi-solid (0.15% agar-agar) JNFb$^-$ medium to test the pellicle formation. Appearance of pellicle formation, if any, was observed after 48 hr of growth at 30^{o}C.

Test of Phosphate Solubilization

Test of phosphate solubilization was performed as per the method of Goldstein (1983), (*Vincent 1970).*

Following medium was prepared;

Table 1. The Endophytic bacteria were isolated from indigenous rice grown by the indigenous people of Thakurgoan Village of Ranchi District, Jharkhand and, important features (based upon the TK of tribal farmers of the region) of the selected varieties and their cultivation pattern are given. (All the information are based on the local tribal knowledge)

Place	Location of the rice field	Latitude and longitude	Rice variety	Land type	Importance
State- Jharakand Country- India	Village- Thakur Goan District- Ranchi	23.52^{oN} 85.17^{0E} at an average elevation of 625m	Sada Gora, Kala Gora, Bala Gora, Kharani (figure 1,2,3, 4)	All the varieties are upland variety but can be grown in low land also and the varieties selected are mostly sown by broadcasting method .	Oldest known rice which can be stored up to 50 years without the use of any other preservatives Used in the preparation of tribal drink Hadia whichis used to cure jaundice. The Hadia prepared from Kharani is known as medicinal Hadia

Solution I: General-purpose agar-agar 20 g, Glucose 10g, NH4Cl 5g,
NaCl 1g, MgSO4.7H2O 1 g, and TDW 1 L, pH 7.2.

Solution II: 5g K2HPO4 in 50 ml TDW.

Solution III: 10g CaCl2 in 100 ml TDW.

These three medium were autoclaved separately and cooled down to about 50^{oC} and then solution II was added to solution I and finally to solution III. Another medium developed by Mehta and Nautiyal (2001) was also employed for testing *P* solubilization. The medium consisted of: (g/L) glucose 10, Ca_3 $(PO4)_2$ 5, MgSO4.7H2O 1, KCl 0.2, NaCl 1, NH4Cl 5 and 2% general purpose agar-agar and TDW 1 L. pH was adjusted to 7.0.
Solid agar-agar medium plates were prepared and streaked with the desired culture and incubated for four days at 30^{oC} in a BOD incubator. The plates were screened for the presence of clearing *z* streak. (Table 3 and Figure 4)

Measurement of pH of Medium Following P Solubilization

Cultures were grown in medium containing tricalcium phosphate at 30^{o}C with shaking at 120 rpm in a shaker (Orbitek LT, Scigenics Biotech. Pvt. Ltd; Chennai) and change in pH of the medium, if any, was tested at 24 hr intervals for six days. Initial pH of the medium was adjusted to 7.0. Estimation of pH was made by Cyber Scan pH/Ion 510 Bench pH/Ion/mV Meter (Eutech Instruments Pte Ltd., Singapore), (Table 5).

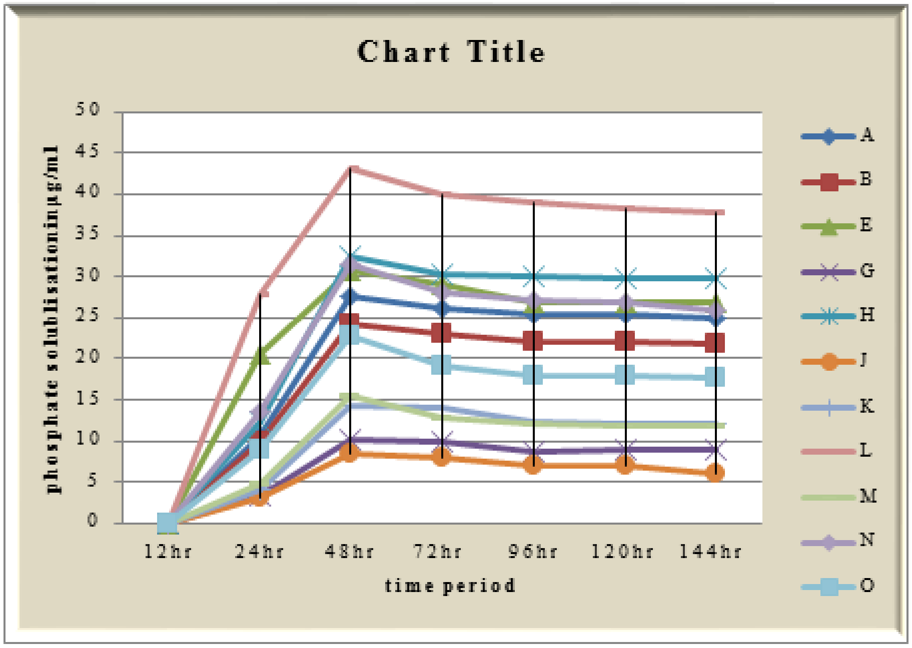
Course Activity of P Solubilization

Once it became evident that majority of the isolates do fix atmospheric nitrogen, it was desirable to assess other beneficial character present, if any, in these isolates. Accordingly, phosphate solubilization test was performed by observing halo zone formation around the growing colonies on medium containing insoluble phosphate (Figure. 4). Out of all the 15 isolates, 11, isolates elicited distinct halo zone formation on solid agar-agar medium indicating the property of mineral phosphate solubilization (MPS). Efficiency of phosphate solubilization was further confirmed by measuring the level of solubilized *P* in the medium.

With a view to study the rate of *P* solubilization activity with growth of culture, time dependent assay was made. It is evident from the data represented in Figure 6 that *P* solubilization activity started after 24 hr of growth and the level increased up to 96 hr in four isolates and thereafter there was no further increase. (Graph 1)

Thin Layer Chromatography

For determination of acid involved in *P* solubilization, culture supernatant of each bacterial isolates grown in modified Nautiyal {18} medium for 3 days, was subjected to thin layer chromatography (TLC). TLC was performed on glass plates (20 x 20 cm) coated with 0.25 mm thick layer of silica-gel 60 G containing 13% $CaSO_4$ (E. Merck, Darmstadt, Germany). The plates were air dried and then activated at 120^{o} for 90 min.20 µl of each concentrated samples were loaded on TLC plate with Hamilton syringe. Simultaneously, 20 µl of gluconic acid, succinic acid,



Graph 1. Time Course Activity of P Solubilisation

malic acid and citric acid (10mg/ml) were also loaded to serve as standard. After drying the spots, the plate were developed in a solvent mixture containing butanol: acetic acid: water in the ratio 12:13:5. Normally the solvent front was allowed to move 16-18 cm from the origin. The chromatogram was dried overnight at 37^{oC} in a hot-air oven. Presence of acid was detected by spraying bromocresol green (50 mg/ml in 50% ethanol) on TLC plate. Appearance of yellow color indicated the presence of gluconic acid. (Figure`5)

RESULT AND DISCUSSION

Isolation of putative diazotrophic endophytes from different plants

Indigenous rice varieties Sada Gora, Bala Gora, Kala Gora and Kharani were chosen for isolation of endophytic diazotrophic bacteria. The selection of these rice varieties for isolation of endophytic bacteria was based on the level of available nitrogen in three particular fields from where plants were collected. Different parts of plant such as roots, culm and seed were used for the screening of endophytic bacteria .When macerate of different dilutions of surface sterilized plant's parts such as roots, culm were plated on Nutrient Agar or $JNFb^{-}$ medium, a number of discrete colonies of bacteria appeared after 3-4 days of growth. The appearance of bacterial colonies was observed in the macerate of all the parts of plants such as roots and culm. The highest number of bacterial isolates based on differences in morphological difference was observed in the selected rice variety (Table 2).

Test of Phosphate Solubilization

Phosphate solubilization test was performed by observing halo zone formation around the growing colonies on medium containing insoluble phosphate. Out of all the 15 isolates, 11 isolates elicited distinct halo zone formation on solid agar-agar medium indicating the property of mineral phosphate solubilization (MPS). Efficiency of phosphate solubilization was further confirmed by measuring the level of solubilized *P* in the medium (Table 4).

DISCUSSION

The search for natural endophytes associated diazotrophs with rice of and other economically important crop plants such as wheat is considered very promising in view of promoting higher yield (*Ladha and Reddy 2000)*. Indigenous rice varieties as well as some cultivated varieties are considered as excellent hosts to identify endophytes that may impact agricultural crop production. In present investigation, different varieties of indigenous rice (O*ryza sativa*), has been selected in view of earlier reports that some rice varieties, cultivated with little or no inputs of chemical nitrogen fertilizers, harbor diazotrophic endophytic bacteria (*Gaur 2003)*. The selected rice variety (figure 1-4) shows their growth in natural condition in water stress condition without the application of any fertilizer and insecticides. The various parts of the rice variety from which the bacterial isolates were isolated after sterilization. Sterilization by treating with ethanol and Chloramine *T* was effective in removing

Table 2. Isolation of putative endophytic diazotrophic bacteria from different parts of selected rice variety.

Plant/Variety	Part/Organ	Designated Isolates	Total No. of Isolates
Bala Gora	Root Stem	BGS2, BGS3, BGS6 BGR7, BGR5, BGR10, BGR12	3 4
Sada Gora	Root Stem	--- SGS1, SGS2, SGS3	0 3
Kala Gora	Root Stem	KGR2 KGS2	1 1
Karhaini	Root Stem	KHR1,KHR2 KHS1	2 1

Table 3. Putative endophytic diazotrophic bacteria showing +ve phosphate solubilisation

Sl.no	Bacterial name	Endophytes	JNFb -	Phosphate solubilization
1	BGS2	Y	+	+
2	BGS3	Y	+	+
3	BGS6	Y	+	+
4	BGR5	Y	+	_
5	BGR7	Y	+	_
6	BGR10	Y	+	+
7	BGR12	Y	+	_
8	KHS1	Y	+	+
9	KHR1	Y	+	+
10	KHR2	Y	+	+
11	SGS1	Y	+	_
12	SGS2	Y	+	+
13	SGS3	Y	+	+
14	KGS2	Y	+	+
15	KGR2	Y	+	+

most of the microbes from the plant surface. Absence of any growth on nutrient media spread with last wash ascertained proper elimination of rhizoplanic bacteria. Diverse diazotrophic bacteria were then isolated from the surface sterilized plant parts on medium JNFb$^-$ devoid of any organic or inorganic nitrogen sources. Continuous

Table 4. *P* -Solubilising Isolates from Different Parts of Selected Indigenous Rice Variety

Root Endophytic Diazotropic Isolates Showing Phosphate Solubilisation				
Growth Medium	Bala Gora (BG)	Kharanai (KH)	Sada Gora (SG)	Kala Gora (KG)
JNFb- Liquid	3	2	0	1
Stem Endophytic Isolates Showing Phosphate production				
Growth Medium	Bala Gora (BG)	Kharanai (KH)	Sada Gora (SG)	Kala Gora (KG)
JNFb-	2	1	1	1

Table 5. Time course activity of phosphate solublisation by different isolates

Time Period	Phosphate Solublisation in µg/ml										
	A	B	E	G	H	J	K	L	M	N	O
12hr	0	0	0	0	0	0	0	0	0	0	0
24hr	10.66	9.9	20.68	3.33	12.45	3.16	4.05	27.97	4.86	13.58	8.89
48hr	27.6	24.3	30.75	10.2	32.4	8.5	14.25	43.22	15.6	31.5	22.8
72hr	26.1	22.9	28.99	10	30.39	7.98	14	40.09	12.9	28	19.11
96hr	25.55	22.06	27.01	8.8	30.01	6.99	12.37	38.99	12	27.08	18.03
120hr	25.5	21.99	26.99	8.89	29.9	6.99	12.08	38.34	11.87	27	18
144hr	25.05	21.9	26.9	8.85	29.9	6.87	12	37.77	11.8	26.9	17.56

(A-BGS2, B-BGS3, C-BGS6, F-BGR10,H-KHSI I-KHR1,J-KHR2,L-SGS2 M-SGS3 ,N-KGS2 O-KGR2)

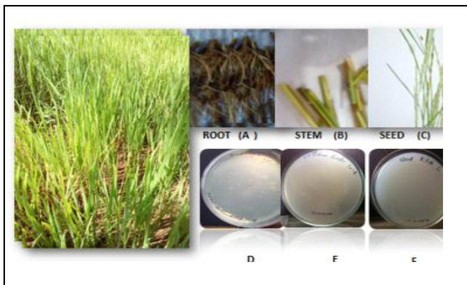


Figure 1. KARHANI rice (a-f) Showing rice growing in dry part from which endophytes were Isolated Gr growth of bacteria on JNFb-

Figure 2. BALA GORA (a-f) feilds showing rice growing in dry parts from which endophytes were Growth of bacteria on JNFb-

Figure 3. SADA GORA (a-f) feilds showing rice growing in dry fields parts from which endophytes were Growth of bacteria on JNFb-

Figure 4. Feilds showing rice growing in dry fields showing rice growing in dry a-: Growth of bacteria on JNFb

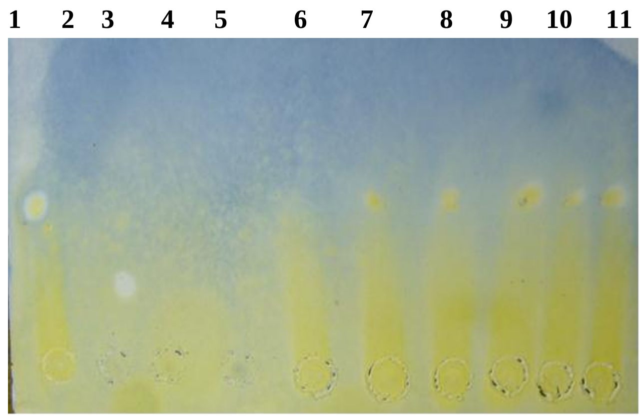


Figure 5. TLC of acid produced as a result of P solubilization
Lane: 1- Gluconic acid.2 Succinic Acid
Lanes (3-11) supernatant of various endophytic diazo trophic bacterial isolates from various rice

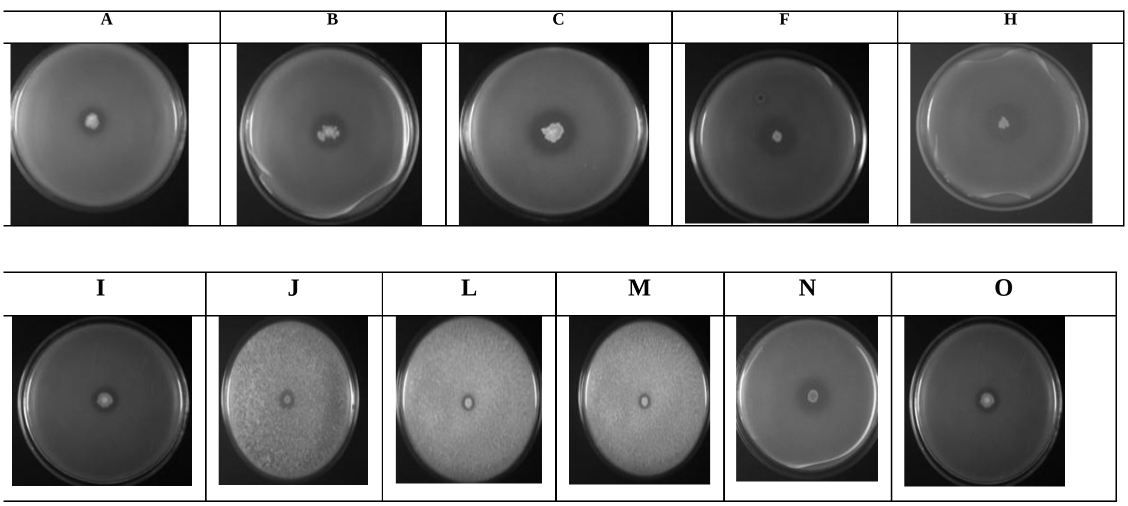


Figure 6. Phosphate solubilizing activity by various bacterial isolates.
(A-BGS2, B-BGS3, C-BGS6, F-BGR10,H-KHSI I-KHR1,J-KHR2,L- SGS2 M-SGS3 ,N-KGS2 O-KGR2)
P solubilization test was made following growth of cultures in Goldstein medium
P solubilized was estimated after 4 days of growth. Results are based on average of two experiments performed under identical conditions.

growth on JNFb$^-$ for several generations by repeated sub culturing confirmed the diazotrophic nature of the isolates. Bacterial isolates constantly growing on N$^-$ medium were further subjected to primary characterization. Higher number of isolates from stem in comparison to root parts suggests presence of more translocation of diverse microflora from roots stem during the growth period. Similar finding was also observed in few varieties of rice by (Hilda and Fraga 1999).

All the bacterial isolates were able to fix atmospheric nitrogen; it was desirable to assess other beneficial character present, if any, in these isolates. Accordingly, phosphate solubilization test was performed by observing halo zone formation around the growing colonies on medium containing insoluble phosphate (Figure. 4). Out of all the 15 isolates, 11 isolates elicited distinct halo zone formation on solid agar-agar medium indicating the property of mineral phosphate solubilization (MPS).

Most of the soil in cultivation in various parts of Jharkhand has 35 kg/ha of phosphorous, primarily in the unavailable form of precipitated tri-calcium phosphate ($Ca_3(PO)_2$) and only 2-3 ppm phosphorous is available to rice (Mano and Hisao 2008). About 66% of the soils in Jharkhand are deficient in available phosphate. Gramnegative bacteria are well known to dissolve bound phosphates such as calcium triphosphate, hydroxyapatite and rock phosphate, and enhance the availability of phosphorous for microbial and/or plant growth (Son et al., 2005). So, the solubilization of precipitated phosphates by rhizobacteria in situ would enhance phosphate availability to rice and other crops in these soils, thus representing possible mechanisms of PGP (Plant growth promotion) under field conditions. In present investigation the extracellular solubilization of precipitated tri-calcium phosphate in vitro showed that several isolates were positive for this property. Solubilizing bacteria are severely influenced under phosphate stress condition (Cankar et al., 2005).

However, level of *P* solubilization by these bacteria varied with different nitrogen sources. With response to particular *N* sources, two mechanisms in bacteria have been suggested. In one mechanism, the presence of ammonium in growth medium results in the development of inorganic acid by proton exchange mechanism. Another mechanism does not require presence of ammonium and involves excretion of organic acid metabolites (Boddey 1995). Our results showed *P* solubilization in both ammonium and nitrate suggesting different mechanism of *P* solubilization in bacteria.

Results of time course activity of *P* solubilization clearly indicate that the compounds responsible for *P* solubilization activity are released in stationary phase of growth of bacteria. In addition to physical conditions of soil, other factors such as temperature, moisture, aeration and humic acid, etc do influence the MPS activity of bacteria (Nautiyal et al., 2000)].

Involvement of acid production in microbial *P* solubilization process is evident in several studies (Xalxo 2008) sharp decreases in pH of the medium with growth of culture indicate production of acid. The change in pH value showed an inverse correlation with the soluble *P* concentration (Lakraet al., 2010). In this study, analysis of culture supernatant depicted the presence of organic acid chiefly glycolic acid. Thus, *P* solubilization process by the isolates used in present investigation is organic acid mediates. Organic acids have been implicated to chelate the cationic counterpart of *P* ion and release soluble *Pi* in the medium (Ramaiah 1953). Analysis of culture filtrates of many MPS bacteria has shown the production of mono, di and tricarboxylic acids. Although, solubilization of tri-calcium phosphate is known to occur even in the absence of release of organic acid (Dang et al., 2013).

Application of endophytic diazotrophic bacteria to crop plants has highly been acknowledged to attain a sustainable cultivation. In view of this, isolation of efficient growth promoting endophytic bacteria is required. Since, there are probability of occurrence of diazotrophic endophytes in indigenous rice plants growing in little or no nitrogen input condition (Souza et al., 2014), from properly surface sterilized roots, culms and of selected varieties of rice,15 diazotrophic bacterial isolates were selected on the basis of morphological differences on solid agar-agar plates. Nitrogen-fixing bacterial isolates were enriched and maintained on medium JNFb$^-$ devoid of any organic or inorganic nitrogen sources. Highest bacterial population was found in roots of Kaharani followed by Kala Gora rice variety. The isolates showed *P*-solubilization and this potential along with other PGP character can be the potential bio fertilizer in the sustainable rice cultivation in phosphate deficient land and can help in bringing in digenous rice cultivation into mainstream agriculture from marginal cultivation to an extent (Sarker et al., 2014).

CONCLUSION

The potential of endophytic diazotrophic bacteria to increase crop growth and yields are greatly acknowledged. In addition to biological nitrogen fixation, they exert other beneficial properties through *P* solubilization, growth promoting or hormonal substances and disease control (Gaur 1990). Primary screening for these growth promoting activities in putative endophytes clearly demonstrates the presence of plant growth promoting activity in these isolates. Selection was made on the basis of high nitrogen fixing ability along with *P* solubilization Phosphorous limits crop productivity as it is involved in many essential processes like cell division, photosynthesis, sugar breakdown, transfer of energy and nutrient uptake. Biologically mediated processes such as mineralization and mobilization largely control the availability of soil phosphorous (Gaur 1990). Mineral phosphate solubilization (MPS) phenotype has been of great interest due to action of *P* solubilizing bacteria on poorly soluble calcium phosphates and other insoluble form of phosphorus in soil. They can enhance the availability of *Pi* for microbial and/or plant growth. Highest phosphate solubilization activity was exhibited by SGS2, which suggests it as a strong phosphate solubilizer isolate and can be used in field condition as a bio fertilizer in soil where level phosphorous is low.

Results of time course activity of *P* solubilization clearly indicate that the compounds responsible for *P* solubilization activity are released in stationary phase of growth of bacteria [1][th]. In addition to physical conditions of soil, other factors such as temperature, moisture, aeration and humic acid etc do influence the MPS activity of bacteria [23(b)]. Therefore performance of P solubilizing bacteria is severely influenced under stress condition [2][ds]. From the above discussion it is clear that out of 15, putative endophytic diazotrophic from selected ethno medicinal, indigenous rice of Jharkhand bacteria, 11 were able to solubilize phosphate. The solubilization was accompanied by the production of acid as recorded with decrease in pH of the medium. The screening of the putative endophytic diazotrophic bacteria from the rice is one of the initial work related to the indigenous rice of the state and further characterization and evaluation on the growth and yield of the rice will lead to the selection of efficient *P*-solubilizing, N2 fixing bio fertilizer for sustainable rice cultivation in P deficient agricultural tribal rice farming in Jharkhand.

Acknowledgement

We express our deep sense of gratitude to Prof. Ashok Kumar, School of Biotechnology, Institute of Science, Banaras Hindu University, Varanasi, for his constant support throughout the work. We are thankful to Prof. A.K Chaudhary, Head Department Of Botany, Ranchi University, Ranchi And Dr Jyoti Kumar, Professor, Department Of Botany, Ranchi University, for their suggestions and constant encouragement the preparation of the manuscript . We are very thankful to Mr. R Clement, School Of Biotechnology, BHU. Varanasi, for helping us in conducting our research experimental Work. We are also thankful to Dr Shalini Lal Assistant Professor, Dr Shyma Prasad Mukherjee University Ranchi and Dr Smriti Prabha for critically going through the manuscript and also giving their valuable inputs during work planning and co -ordination.

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